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Studies of oxidative stress in cellular systems

The interaction of monocytes and erythrocytes

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^{1}H spin echo NMR spectroscopy is used to follow the interaction of intact and viable erythrocytes and monocytes obtained from different sources in mixed cultures. After a lag time (270 min) erythrocyte glutathione is observed to become more oxidised. This result is believed to occur as a consequence of monocyte activation generating hydrogen peroxide or hypochlorous acid, which is targeted at the erythrocyte. The red cell in turn employs its sulphydryl system as an anti-oxidant defence.

Erythrocyte; Monocyte; Glutathione; Oxidative stress; NMR spectroscopy; Immune response

1. INTRODUCTION

Uncontrolled oxidation of tissues is a facet of many disease processes [1–3]. Current belief is that this results from the generation of either toxic species such as hydrogen peroxide or free radicals such as the hydroxyl radical or the superoxide radical anion [3]. There are a number of common chemical targets for these oxidants which can be assayed directly through the determination of such markers as lysate thiol [4] or the products of lipid peroxidation [2]. Although oxidative bursts by the monocyte can be observed using fluorescent probes [5], the events leading up to this situation and the chemistry associated with the attack of a cell such as the monocyte on a foreign body have as yet not been observed in-situ in a kinetic manner.

Using NMR spectroscopy it is possible to observe changes in the biochemistry of the intact cell during an oxidative insult [6] such as that which occurs with hydrogen peroxide [7,8] or anthracyclines [9]. As such it was thought that it might be possible, using ^{1}H spin echo NMR spectroscopy, to observe aspects of cellular biochemistry which occur when an erythrocyte is attacked by a defence cell such as the monocyte.

2. EXPERIMENTAL

2.1. *Isolation of erythrocytes*

Whole blood (10 ml) was collected in heparinised anti-coagulant tubes from healthy normal volunteers. The whole blood was centrifuged at 3,000 rpm (1,000 × *g*) for 10 min at 4°C and the plasma and buffy coat drawn off with a Pasteur pipette. 0.5 ml of the isolated red cell pellet obtained as above was washed once in ^2H_2O/NaCl (0.154 M)/Na_2HPO_4 (0.125 M) to facilitate oxygen uptake and twice in ^2H_2O saline (0.154 M NaCl).

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2.2. *Isolation of monocytes*

Whole blood (30 ml) was collected in EDTA coated glass tubes. The whole blood (10 ml) was mixed with 5% Dextran T500 (1 ml) in sterile conical tubes. The blood was left for 30–45 min to allow the erythrocytes to settle out, leaving the leukocyte rich plasma (LRP) layer on top. The LRP layer was then gently layered onto Nycodenz (monocyte) (3 ml) in a second conical tube. Tubes were centrifuged at 1,500 rpm for 15 min at 4°C. The monocytes collect at the interface between the Nycodenz and the plasma-dextran are drawn off with a Pasteur pipette and washed three times in Hanks solution (calcium and magnesium free, 1,500 rpm at 4°C). Prior to use the monocytes were washed three times in ^2H_2O/saline (0.154 M NaCl). Samples were obtained from volunteers with different blood groups to ensure that they were not compatible.

2.3. *NMR spectroscopy*

0.5 ml of packed erythrocytes were placed in a 5 mm NMR tube and a monocyte pellet obtained from a 10 ml blood sample added. This is an effective 10-fold increase over the natural monocyte concentration in the blood.

NMR spectra of the erythrocyte suspensions were recorded using a Carr–Purcell–Meiboom–Gill sequence $90°-t(-180°-t)_n$ with a delay time (t) of 60 ms and only one repetition (total delay 120 ms). A Bruker AMX 400 MHz spectrometer was used to record all spectra. Samples were maintained at 20°C during data collection and the data from 2,000 complete pulse sequences were accumulated for each spectrum. The free induction decay (FID) was collected in 8K of memory (acquisition time 90 min) to which a 0.5 Hz line broadening function was applied, prior to zero filling to 64K and Fourier transformation. The 90° pulse was generated with a 12.5 μs pulse width. The residual water was eliminated from the spectrum by presaturation during relaxation delay (58 dB; D1 = 0.5 s).

NMR spectra of the isolated intact monocyte suspensions were recorded using a Carr–Purcell–Meiboom–Gill sequence $90°-t(-180°-t)_n$ with a delay time (t) of 0.32 ms with 422 repetitions (total delay 135 ms). It was necessary to use a larger blood volume (25 ml) to provide enough cells (10^7) for the sample and 4,000 scans to obtain a data set. The other acquisition and processing parameters are as given above.

2.4. H_2O_2 production by monocytes

The ability of the monocyte preparations to secrete hydrogen peroxide was measured spectrophotometrically using an assay based on the horseradish peroxidase oxidation of Phenol red [10]. 1 ml aliquots of monocytes (10^6 cells) were incubated with different species as described in Table I for 1 h at 37°C. The samples were then centrifuged at 1,000 r.p.m. for 5 min at 37°C to obtain a cell free supernatant. 100 μl of the supernatants were added to 200 μl of a solution containing NaCl (0.140 M), $CaCl_2$ (1 mM), phosphate buffer (pH 7.0), glucose (5.5 mM), Phenol red (0.56 mM) and 19 μl/ml of horseradish peroxidase and incubated in the dark at 37°C for 1 h. The reaction was halted by the addition of sodium hydroxide (10 μl, 3 M). The oxidation of Phenol red by the hydrogen peroxide generated from the monocytes was assessed by measuring the absorbance at 600 nm. Known concentrations of hydrogen peroxide were used to produce a calibration curve.

3. RESULTS AND DISCUSSION

The spin echo NMR spectrum of the erythrocyte is dominated by the resonances assigned to glutathione [7,8,11]. Of paramount importance to this study are the resonances g_2 and g_4, which act as internal markers for the oxidation state of this species. When g_2 is strongly negative it indicates that the intracellular pool of glutathione is mainly in the reduced state [11]. As glutathione is oxidised the resonances associated with g_2 split and diminish in signal intensity [11] (Fig. 1).

In contrast, the monocyte spectrum (Fig. 2) has only a limited pool of NMR visible glutathione. The glycyl signal (g_1) can just be observed in a pure monocyte suspension but at the cell concentration employed in the mixed sample the g_2 and g_4 resonances derived from the monocyte would not be observable above the noise. As such, in the mixed cell sample the glutathione resonances can be assigned solely to the erythrocyte (Fig. 1).

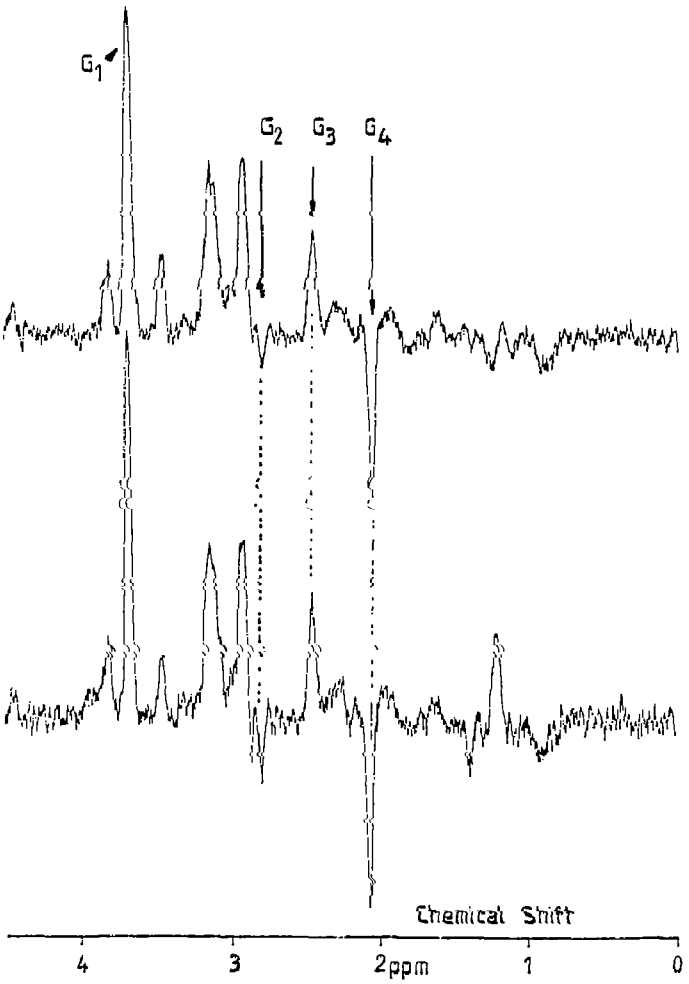

Fig. 1. The 400 MHz ^1H spin echo NMR spectra of the human erythrocyte prior to (below) and after (above) mixing with the monocyte. Oxidation of erythrocyte glutathione is evidenced by the alteration in signal intensity of the g_2 beta-cysteinyl resonance. Resonance assignments follow previous studies [7,8,10].

Table I

The production of hydrogen peroxide from monocytes stimulated by native and foreign erythrocytes and the tumour promoter phorbol myristic acid PMA as assessed by the horseradish peroxidase assay

Contents of cell cultures	nmol H_2O_2 produced
Erythrocytes + saline control	0.0
Monocytes + PMA (10^{-12} g/ml)	30
Monocytes + PMA (10^{-9} g/ml)	35
Monocytes + PMA (10^{-6} g/ml)	72
Monocytes + 100 μl saline (control)	10
Monocytes + native erythrocytes (50 μl)	22
Monocytes + native erythrocytes (100 μl)	40
Monocytes + native erythrocytes (500 μl)	18
Monocytes + 100 μl saline (control)	18
Monocytes + foreign erythrocytes (100 μl)	30
Monocytes + 100 μl saline (control)	10

On treatment of the erythrocytes with the monocytes there is no immediate change in the spectra. After 270 min an alteration in the g_2/g_4 ratio, consistent with the oxidation of erythrocyte glutathione is observed (Fig. 1,3). It is known that the interaction of peroxides with intact cells is rapid [7,8,11]. As such, the kinetics of the process, especially the delay in response is probably derived from the response of the monocyte.

During the NMR experiment, the cells are in a severe environment with many of the small molecules and proteins (albumin, globulin) present in plasma missing. As such, cellular recognition processes may be expected to be down-regulated. However, once the monocytes become activated the protective response to chemical attack on the erythrocytes is observed (Fig. 3). There is no appreciable change in the control experiment using

erythrocytes suspensions without any peripheral white cells present and the spectra remain stable over a longer time scale than that used in these experiments [11]. When monocytes are re-incubated with their parent erythrocytes there would seem to be some response. This is thought to result from the activation of the cells during handling, which will be amplified as a consequence of the 10-fold increase in the monocyte concentration in the suspension. However, the oxidative insult does not produce the same degree of intracellular response to that observed for the mixture of monocytes with foreign erythrocytes.

The monocyte is capable of generating strong oxidants such as hydrogen peroxide and, via myleo-peroxidase hypochlorous acid. Using the horseradish peroxidase assay [10] it is possible to make a comparison between the activation of the monocyte in the presence of native erythrocytes, foreign cells and the tumour promoter phorbol myristic acid (PMA). As compared to a saline control, PMA induces the production of the largest amount of hydrogen peroxide, almost twice that observed for the two erythrocyte suspensions (Table 1). This value represents the maximal amount of hydrogen peroxide which can be generated in the 1-h incubation period in the absence of any anti-oxidant. Incubations of monocytes with native and foreign erythrocytes generate similar amounts of hydrogen peroxide in each case as compared to their respective saline controls. The stimulant in this experiment are the erythrocytes. As shown in Fig. 3, erythrocytes are also efficient anti-oxidant species which suggests that the horseradish peroxidase assay only detects the surplus hydrogen peroxide generated after some has reacted with the cell. The discrepancy between the PMA incubated monocytes and the monocytes incubated with foreign erythrocytes in the horseradish peroxidase assay probably explains the effects observed in Fig. 3.

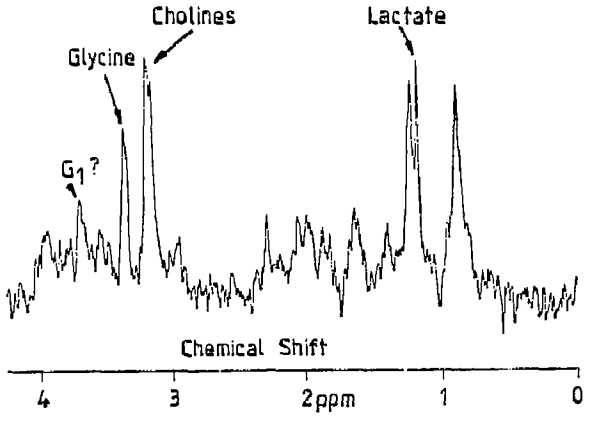

Fig. 2. The 400 MHz ^1H spin echo NMR spectrum of the human monocyte. The resonances have been tentatively assigned using data from the erythrocyte. Of major importance to this study is the failure of the resonances g_2–g_5 derived from glutathione to rise above the signal to noise. Sample size 10^7 cells/0.5 ml.

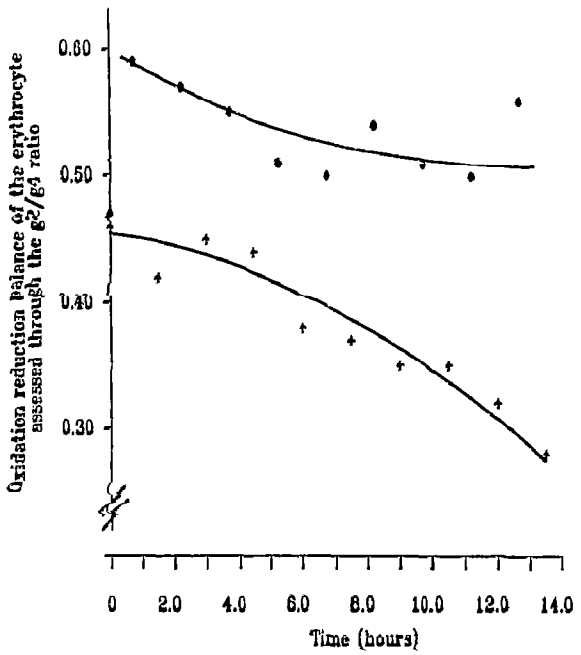

Fig. 3. The oxidation of erythrocyte glutathione by the monocyte: (◆) incubated with native erythrocytes as a control; (+) incubated with foreign erythrocytes. Rapid change in the oxidation reduction balance is observed only after 270 min.

This simple experiment presents the prospect of investigating further intracellular signalling and the screening of immune suppressant compounds and anti-oxidants. It also re-inforces the concept that the erythrocyte is an important circulating anti-oxidant species.

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